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(57) Abstract

The subject inventions provide a vaccine which comprises *vif* HIV, a nucleic acid molecule encoding *vif* HIV, a nucleic acid molecule encoding a vif antisense oligonucleotide and a nucleic acid molecule encoding a non-functional mutant Vif protein capable of inhibiting the function of wild-type Vif. The subject inventions are to be used to reduce the likelihood of an individual becoming HIV infected or to treat an HIV infected subject.

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# HIV *vif*-RELATED COMPOSITIONS, AND PROPHYLACTIC AND THERAPEUTIC USES THEREOF

This application is a continuation-in-part of U.S. Serial No. 08/110,226, filed August 20, 1993 the contents of all of which are hereby incorporated by reference into the subject application.

## Background of the Invention

Throughout this application, various publications are referenced by Arabic numerals. Full citations for these references may be found at the end of the specification immediately preceding the claims. The disclosure of these publications is hereby incorporated by reference into this application to describe more fully the art to which this invention pertains.

The rate limiting steps in the infection of $CD4^+$ T cells with HIV-1 deficient in the viral infectivity factor, *vif*, are unknown. It has been previously shown that *vif*-deficient HIV-1 fuses efficiently with target T cells in vitro but that subsequent viral replication and spread are slow (38).

The infectivity factor of HIV-1, *vif*, is a 23 kDa protein which facilitates HIV-1 infection in cultured T cell lines and is required for infection of normal peripheral blood lymphocytes *in vitro* (1, 8, 9, 10, 18, 22, 24, 29, 33, 34). The *vif* open reading frame is conserved among animal lentiviruses (25, 35), and the majority of HIV-1 infected individuals have serum antibodies to *vif* (2, 16, 20, 31), suggesting that this protein is important in natural virus infection. It is believed that *vif* is both synthesized and active at a late phase of the viral life cycle (11, 14, 19, 32), and that it enhances the infectivity of progeny virus (10). Transmission of virus

by cell-to-cell contact also requires the activity of *vif* (9, 22, 30). Nonetheless, the mechanism of action of *vif* is unknown. *vif* has no detectable effect on proviral DNA transcription, translation, or virus secretion (1, 9, 18, 22, 33). A recent report proposed that *vif* plays a role in the modification of HIV-1 envelope glycoproteins (12), but this finding has been challenged (8, 10). The basis for the enhanced infectivity of HIV-1 particles produced in the presence of *vif* is also unclear. Some reports postulate that *vif*-deficient HIV-1 contains a high proportion of viral particles which are defective at entry (8, 34), and results of others indicate that *vif* is required for competence in both viral entry and viral spread (9,10).

As previously demonstrated, HIV-1/N1T-E, the *vif*-defective molecular clone of the N1T virus (29, 30), maintains the capacity to fuse with CEM cells, but is delayed in the subsequent synthesis of viral RNA and proteins, resulting in a slow non-cytopathic infection (22).

The subject invention provides a vaccine, compositions and prophylactic and therapeutic methods which exploit the vital role of *vif* in the HIV life cycle.

## Summary of the Invention

The subject invention provides a vaccine which comprises an effective immunizing amount of *vif*⁻ HIV and a pharmaceutically acceptable carrier.

The subject invention also provides a method of reducing the likelihood of a non-HIV-infected subject's becoming infected with HIV which comprises immunizing the non-HIV-infected subject with the vaccine of the subject invention so as to thereby reduce the likelihood of the subject's becoming infected with HIV.

The subject invention further provides a composition which comprises an effective amount of a nucleic acid molecule encoding *vif*⁻ HIV capable of being expressed in a suitable host cell, and a pharmaceutically acceptable carrier.

The subject invention further provides a method of reducing the likelihood of a non-HIV-infected subject's becoming infected with HIV which comprises administering to the non-HIV-infected subject an amount of the composition of the subject invention effective to reduce the likelihood of the subject's becoming infected with HIV.

The subject invention further provides a composition which comprises an effective amount of a recombinant non-HIV virus capable of infecting a suitable host cell, said recombinant virus comprising a nucleic acid molecule encoding *vif*⁻ HIV and capable of being expressed in the suitable host cell, and a pharmaceutically acceptable carrier.

The subject invention further provides a method of

reducing the likelihood of a non-HIV-infected subject's becoming infected with HIV which comprises administering to the non-HIV-infected subject an amount of the composition of the subject invention effective to reduce the likelihood of the subject's becoming infected with HIV.

The subject invention further provides a composition which comprises an effective amount of a recombinant non-HIV virus capable of infecting a suitable host cell, said recombinant virus comprising a nucleic acid molecule (a) encoding an anti-sense oligonucleotide molecule capable of specifically binding to an mRNA molecule encoding HIV *vif* protein, at the portion thereof encoding the HIV *vif* protein, so as to prevent translation of the mRNA molecule, and (b) capable of being expressed in the suitable host cell, and a pharmaceutically acceptable carrier.

The subject invention further provides a method of reducing the likelihood of a non-HIV-infected subject's becoming infected with HIV which comprises administering to the non-HIV-infected subject an amount of the composition of the subject invention effective to reduce the likelihood of the subject's becoming infected with HIV.

The subject invention further provides a method of treating an HIV-infected subject which comprises administering to the HIV-infected subject an amount of the composition of the subject invention effective to treat the subject.

The subject invention further provides a method of treating an HIV-infected subject which comprises: (a) obtaining a sample of hematopoietic stem cells from the HIV-infected subject; (b) culturing the sample <u>in vitro</u>,

thereby producing cultured hematopoietic stem cells; (c) introducing into the cultured hematopoietic stem cells so produced a nucleic acid molecule (a) encoding an anti-sense oligonucleotide molecule capable of specifically binding to an mRNA molecule encoding HIV *vif* protein, at the portion thereof encoding the HIV *vif* protein, so as to prevent translation of the mRNA molecule, and (b) capable of being expressed in the cultured hematopoietic stem cells; and (d) introducing the resulting cultured hematopoietic stem cells into the HIV-infected subject under conditions permitting the resulting cultured hematopoietic stem cells to reconstitute the HIV-infected subject's hematopoietic system, so as to thereby treat the HIV-infected subject.

The subject invention further provides a composition which comprises an effective amount of a recombinant non-HIV virus capable of infecting an HIV-infected cell, said recombinant virus comprising a nucleic acid molecule (a) encoding a non-functional mutant HIV *vif* protein capable of competitively inhibiting the function of HIV *vif* protein in the HIV-infected cell, and (b) capable of being expressed in the HIV-infected cell, and a pharmaceutically acceptable carrier.

Finally, the subject invention provides a method of treating an HIV-infected subject which comprises administering to the HIV-infected subject an amount of the composition of the subject invention effective to treat the HIV-infected subject.

## Brief Description of the Figures

Figure 1

Schematic representation of genomic maps of viral clones used in this work. The recombinant viruses were constructed as described (30). Restriction enzyme sites: A, *Apa*I; E, *Eco*RI; N, *Nde*I; Sa, *Sal*I; S, *Stu*I.

Figure 2

HIV-1 DNA synthesis in *vif*-deficient and *vif*$^+$ virus-infected MT-2 cells as analyzed by PCR for the *gag* region. Cells were infected with N1T-A or KS282 as described in the text. Numbers in parentheses indicate viral dose (pg p24 per cell). At the designated time points, cell samples were removed and PCR was performed as described in the text. Each system contained the equivalent of 10,000 cells as determined by β-globin DNA content (shown in the lower panels under viral DNA panels). The ACH-2 panel shows *gag* region DNA amplification using lysates from the designated number of ACH-2 cells. The *gag* primers were SK38 and SK39 (26), the β-globin primers were PC04 and GH20 (4), the number of PCR cycles was 25. Amplified DNA was resolved by 1.5% agarose gel electrophoresis, followed by Southern blotting and hybridization (3) with the $^{32}$P-labeled SK19 (26) and β-globin primers and probe were custom-synthesized. Virus production as determined by the levels of p24 core antigen in culture supernatants on day 5 after infection was, in ng p24/ml, N1T-A (0.08): 157; N1T=A (0.39): 261; KS282 (0.45): 0.6; KS282 (2.24): 3.8.

Figure 3

HIV-1 DNA synthesis in *vif* and *vif*$^+$ HIV-1-infected MT-2 cells as described in the text and in the legend to Figure 2. N1T-A ice and KS282 ice designate systems in which cells were incubated with the respective viruses

for 1 hour at 0°C, then analyzed as described; N1T-A h.i. and KS282 h.i. designate cells infected with heat-inactivated viruses (30 min at 56°C); A+282: pN1T-A and PKS282 plasmid DNA at a 1:1 ratio. The *vif* region primers were VF5071 (5'GGC AAG TAG ACA GGA TGA GGA 3') and VF5411 (5' TAA GGC CTT TCT TAT TGC AGA 3'); the number of PCR cycles was 30, and the internal oligonucleotide probe used for hybridization was VF5282 (5' GGG TCA GGG AGT CTC C3'); all other conditions were as for the *gag* amplification.

Figure 4

Genomic DNA sequence of *vif* protein (SEQ ID NO:2).

Figure 5

Genomic DNA sequence of *vif*-E mutant protein (SEQ ID NO:1).

## Detailed Description of the Invention

The subject invention provides a vaccine which comprises an effective immunizing amount of *vif*⁻ HIV and a pharmaceutically acceptable carrier. HIV includes, for example, HIV-1.

As used herein, "effective immunizing amount" means an amount of *vif*⁻ HIV effective to immunize a non-HIV-infected subject against HIV infection, and may be determined using methods well known to those skilled in the art. As used herein, "immunizing" means administering a dose of the vaccine to a subject so as to generate in the subject an immune response against the *vif*⁻ HIV in the vaccine, and thus against HIV.

As used herein, "*vif*⁻ HIV" means a mutant HIV having a genome which does not encode functional *vif* protein and is incapable of reverting to HIV having a genome encoding functional *vif* protein. Examples of *vif*⁻ HIV include, but are not limited to, a mutant HIV having a genome wherein the *vif* gene is either partly or totally deleted such that subsequent reversion to normal function is impossible, as well as a mutant HIV having a genome wherein the *vif* gene has multiple point mutations capable of inactivating *vif* protein function such that subsequent reversion to normal function is impossible. The ability of a mutant HIV having a genome which does not encode functional *vif* protein to revert to HIV having a genome encoding functional *vif* protein can be assayed in vitro according to methods known to those skilled in the art.

For example, *vif*⁻ HIV does not have a cytolytic life cycle in several cloned cell lines including, but not limited to, MT-2 leukocytes transformed by HTLV I, primary

macrophages, and CEM T-cell leukemia cells. Any reversion in the aforementioned cells of a mutant HIV having a genome which does not encode functional *vif* protein to HIV having a genome encoding functional *vif* would result in cell death which can be easily scored using plaque assays or other cell death assays known to those skilled in the art. Only mutant HIV having a genome which does not encode functional *vif* protein which cannot revert to the cytopathic HIV phenotype after extended growth on these cells is termed "*vif*⁻ HIV". The terms "*vif*⁺ HIV" and "vif-expressing HIV" are used herein synonymously with the term HIV.

Pharmaceutically acceptable carriers are well known to those skilled in the art and include, but are not limited to, 0.01-0.1M and preferably 0.05M phosphate buffer or 0.8% saline. Additionally, such pharmaceutically acceptable carriers may be aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like. Preserva-tives and other additives may also be present, such as, for example, antimicrobials, antioxidants, chelating agents, inert gases and the like.

The subject invention also provides a method of reducing the likelihood of a non-HIV-infected subject's becoming infected with HIV which comprises immunizing the non-HIV-infected subject with the vaccine of the subject

invention so as to thereby reduce the likelihood of the subject's becoming infected with HIV.

As used herein, reducing the likelihood of a subject's becoming infected with HIV means reducing the likelihood of the subject's becoming infected with HIV by at least two-fold. For example, if a subject has a 1% chance of becoming infected with HIV, a two-fold reduction in the likelihood of the subject's becoming infected with HIV would result in the subject's having a 0.5% chance of becoming infected with HIV. In the preferred embodiment of this invention, reducing the likelihood of the subject's becoming infected with HIV means reducing the likelihood of the subject's becoming infected with HIV by at least ten-fold.

As used herein, an "HIV-infected subject" means an individual having at least one of his own cells infected by HIV. As used herein, an HIV-infected cell is a cell wherein HIV has been produced. A non-HIV-infected subject means a subject not having any cells infected by HIV. In one embodiment, a non-HIV-infected subject is an HIV-exposed subject. As used herein, an HIV-exposed subject is a subject who has HIV present in his body, but has not yet become HIV-infected. For example, a subject may become HIV-exposed upon receiving a needle stick injury with an HIV-contaminated needle. In another embodiment, the subject is non-HIV-exposed.

As used herein, "subject" means any animal or artificially modified animal capable of becoming HIV-infected. Artificially modified animals include, but are not limited to, SCID mice with human immune systems. In the preferred embodiment, the subject is a human.

The subject invention further provides a composition which comprises an effective amount of a nucleic acid

molecule encoding *vif*⁻ HIV capable of being expressed in a suitable host cell, and a pharmaceutically acceptable carrier.

The "effective amount" of the nucleic acid molecule encoding *vif*⁻ HIV may be determined according to methods known to those skilled in the art.

As used herein, "nucleic acid molecule" includes DNA and RNA. In one embodiment, the nucleic acid molecule is a DNA molecule. DNA includes, for example, cDNA and genomic DNA. The DNA molecule may be a plasmid. In another embodiment, the nucleic acid molecule is an RNA molecule.

The "suitable host cell" in which the nucleic acid molecule encoding *vif*⁻ HIV is capable of being expressed is any cell capable of taking up the nucleic acid molecule and stably expressing the *vif*⁻ HIV encoded thereby. In the preferred embodiment, the suitable host cell is a striated muscle cell or a cardiac muscle cell.

The subject invention further provides a method of reducing the likelihood of a non-HIV-infected subject's becoming infected with HIV which comprises administering to the non-HIV-infected subject an amount of the composition of the subject invention effective to reduce the likelihood of the subject's becoming infected with HIV.

As used herein, administering may be effected or performed using any of the various methods known to those skilled in the art. The administering may comprise administering intravenously. The administering may also comprise administering intramuscularly. The administering may further comprise administering subcutaneously.

As used herein, an amount of nucleic acid molecule encoding *vif*⁻ HIV effective to reduce the likelihood of the subject's becoming infected with HIV can be determined by methods known to those skilled in the art.

The subject invention further provides a composition which comprises an effective amount of a recombinant non-HIV virus capable of infecting a suitable host cell, said recombinant virus comprising a nucleic acid molecule encoding *vif*⁻ HIV and capable of being expressed in the suitable host cell, and a pharmaceutically acceptable carrier.

As used herein, "recombinant non-HIV virus" means a recombinant virus having at least one structural or regulatory element not shared by HIV, which element permits the recombinant non-HIV virus to infect an HIV-infected cell. The use of a recombinant non-HIV virus circumvents the problem of superinfection, which problem prevents HIV from superinfecting an HIV-infected cell. In one embodiment, the recombinant non-HIV virus is a recombinant virus having HIV structural proteins so that the recombinant virus targets $CD4^+$ cells, but containing a non-HIV gene having non-HIV regulatory elements such that the non-HIV gene is not subject to being downregulated when the recombinant virus infects an HIV-infected cell. $CD4^+$ cells include, for example, $CD4^+$ lymphocytes and monocytes. Recombinant non-HIV viruses may be produced according to methods known to those skilled in the art.

The "suitable host cell" is any cell capable of being infected by the particular virus used. For example, papilloma virus's suitable host cell is a cervical cell, and HIV's suitable host cell is a $CD4^+$ cell.

In one embodiment, the recombinant non-HIV virus is a

retrovirus and the nucleic acid molecule is an RNA molecule.

Retroviruses include any RNA virus that uses reverse transcriptase during replication and is capable of incorporating its genome into the host cell genome (e.g., Rous Sarcoma virus, Mouse Mammary Tumor virus and HIV).

The subject invention further provides a method of reducing the likelihood of a non-HIV-infected subject's becoming infected with HIV which comprises administering to the non-HIV-infected subject an amount of the composition of the subject invention effective to reduce the likelihood of the subject's becoming infected with HIV.

In the preferred embodiment, the "administering" is accomplished by intravenous injection.

The subject invention further provides a composition which comprises an effective amount of a recombinant non-HIV virus capable of infecting a suitable host cell, said recombinant virus comprising a nucleic acid molecule (a) encoding an anti-sense oligonucleotide molecule capable of specifically binding to an mRNA molecule encoding HIV *vif* protein, at the portion thereof encoding the HIV *vif* protein, so as to prevent translation of the mRNA molecule, and (b) capable of being expressed in the suitable host cell, and a pharmaceutically acceptable carrier.

In one embodiment, the suitable host cell is a $CD4^+$ cell. In another embodiment, the suitable host cell is a hematopoietic stem cell.

As used herein, "translation of the mRNA molecule" means the translation of the entire *vif* protein-encoding region

of the mRNA molecule.

In one embodiment, the virus is a retrovirus.

The subject invention further provides a method of reducing the likelihood of a non-HIV-infected subject's becoming infected with HIV which comprises administering to the non-HIV-infected subject an amount of the composition of the subject invention effective to reduce the likelihood of the subject's becoming infected with HIV.

As used herein, the amount of recombinant virus comprising anti-sense oligonucleotides effective to reduce the likelihood of the subject's becoming infected can be determined according to methods known to those skilled in the art.

The subject invention further provides a method of treating an HIV-infected subject which comprises administering to the HIV-infected subject an amount of the composition of the subject invention effective to treat the subject.

As used herein, "treating an HIV-infected subject" means reducing in the subject either the population of HIV or HIV-infected cells, or ameliorating the progression of an HIV-related disorder in the subject.

The subject invention further provides a method of treating an HIV-infected subject which comprises: (a) obtaining a sample of hematopoietic stem cells from the HIV-infected subject; (b) culturing the sample <u>in vitro</u>, thereby producing cultured hematopoietic stem cells; (c) introducing into the cultured hematopoietic stem cells so produced a nucleic acid molecule (i) encoding an anti-sense oligonucleotide molecule capable of specifically

binding to an mRNA molecule encoding HIV *vif* protein, at the portion thereof encoding the HIV *vif* protein, so as to prevent translation of the mRNA molecule, and (ii) capable of being expressed in the cultured hematopoietic stem cells; and (d) introducing the resulting cultured hematopoietic stem cells into the HIV-infected subject under conditions permitting the resulting cultured hematopoietic stem cells to reconstitute the HIV-infected subject's hematopoietic system, so as to thereby treat the HIV-infected subject.

Obtaining a sample of hematopoietic stem cells may be accomplished according to methods well known to those skilled in the art. Such methods include, for example, taking a needle sample of bone marrow from a subject. Culturing hematopoietic stem cells may also be accomplished according to methods well known to those skilled in the art.

Methods of introducing nucleic acid molecules into cells are well known to those of skill in the art. Such methods include, for example, the use of viral vectors and calcium phosphate co-precipitation.

Introducing the cultured hematopoietic stem cells into the subject under conditions permitting the cultured cells to reconstitute the subject's hematopoietic system may be accomplished according to methods known to those skilled in the art. For the purposes of this invention, "reconstitute" means to stably grow and either co-exist with or replace non-cultured hematopoietic cells in the subject.

HIV-infected subjects may also have their hematopoietic system reconstituted using hematopoietic stem cells obtained from a suitable donor other than the HIV-infected subject. A suitable donor, as used herein, is

anyone whose hematopoietic cells will not induce graft vs. host or host vs. graft disease in the HIV-infected subject.

The subject invention further provides a composition which comprises an effective amount of a recombinant non-HIV virus capable of infecting an HIV-infected cell, said recombinant virus comprising a nucleic acid molecule (a) encoding a non-functional mutant HIV *vif* protein capable of competitively inhibiting the function of HIV *vif* protein in the HIV-infected cell, and (b) capable of being expressed in the HIV-infected cell, and a pharmaceutically acceptable carrier.

In one embodiment, the recombinant non-HIV virus is a retrovirus.

A "recombinant non-HIV virus capable of infecting an HIV-infected cell" may be produced according to methods known to those skilled in the art. Examples of recombinant non-HIV viruses include, but are not limited to, a recombinant retrovirus having HIV structural proteins and non-HIV regulatory elements. Such a recombinant retrovirus would target $CD4^+$ cells and would not be downregulated due to HIV infection of the cell.

As used herein, a "mutant HIV *vif* protein capable of competitively inhibiting the function of HIV *vif* protein" means a mutant *vif* protein incapable of normal *vif* protein function yet retaining enough structural similarity to *vif* protein (i.e., retaining enough structurally similar binding) to specifically compete with *vif* protein for *vif* protein-binding site(s) in an HIV-infected cell.

The subject invention further provides a method of treating an HIV-infected subject which comprises

administering to the HIV-infected subject an amount of the composition of the subject invention effective to treat the HIV-infected subject.

The amount of recombinant virus comprising a nucleic acid molecule encoding a non-functional mutant HIV *vif* protein effective to treat an HIV-infected subject can be determined according to methods known to those skilled in the art.

The subject invention further provides a method of determining whether an agent is capable of inhibiting *vif* function which comprises (a) simultaneously titrating a cell stably transfected with vif⁻ HIV whose genome also encodes a detectable marker with (i) a vector encoding functional *vif* protein and (ii) an agent of interest, (b) quantitatively determining the amount of detectable marker expressed in the cell, and (c) comparing the amount of detectable marker so determined with a known standard, so as to thereby determine whether the agent is capable of inhibiting the function of *vif* protein. In other words, if the amount of detectable marker does not increase upon simultaneous titration with the vector encoding functional *vif* protein and the agent of interest, but does increase without the addition of the agent, the agent inhibits *vif* function.

The subject invention further provides a composition which comprises an effective amount of an agent capable of inhibiting *vif* function and a pharmaceutically acceptable carrier.

The subject invention further provides a method of reducing the likelihood of a non-HIV-infected subject's becoming infected with HIV which comprises administering to the non-HIV-infected subject a prophylactically effective amount of the composition of the subject

invention.

The subject invention further provides a method of treating an HIV-infected subject which comprises administering to the HIV-infected subject a therapeutically effective amount of the composition of the subject invention.

This invention will be better understood by reference to the Experimental Details which follow, but those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative of the invention as described more fully in the claims which follow thereafter.

**Experimental Details**

A pair of isogeneic *vif*-expressing and *vif*-deficient HIV-1 (vif⁻ HIV) clones were used, pN1T-A and pKS282, respectively (Figure 1). The KS242 and KS243 viral genomes are identical to N1T-A and N1T-E, the previously described wild-type and *vif*-defective molecular clones of the N1T virus (5, 29, 30), but the plasmids carry shorter cellular flanking sequences (30). The genomic DNA sequence of *vif* protein is shown in Figure 4 (SEQ ID NO:2), and the genomic DNA sequence of *vif*-E mutant protein is shown in Figure 5 (SEQ ID NO:1). The viral genome in pKS282 is identical to pN1T-A, except for the 284 bp *Nde*I-*Stu*I fragment of *vif*, which has been exchanged with the corresponding fragment from pKS243 (N1T-E) containing the 35 bp deletion in *vif*, the mutation responsible for the phenotype of N1T-E (Figure 1 and reference 30). KS282 does not encode the 23 kDa *vif* gene product and, as a result, it is slow and non-cytopathic; N1T-A is fast and cytopathic (22, 30). N1T-A (and thus KS282) has functional *vpr* and *nef* genes, but a non-functional *vpu* gene. To prepare viral stocks for infection, CEM-SS cells were electroporated with pKS282 and pN1T-A DNA, supernatants from highly productive cultures were collected, and virus was concentrated to 1% of the original volume. Viral preparations were tested for their HIV-1 p24 antigen content and tested for biological activity in cells. A multiplicity of infection (MOI) of 1 was equivalent to 1pg p24 per cell (37). Since HIV-1 preparations have been shown to carry viral DNA, both as a contaminant and as a component in a small subpopulation of virions (21, 36), virus stocks were filtered through 0.45μm Millipore filters and treated with bovine pancreatic DNase (type IV, Sigma St. Louis, MO) at 1250 U/ml for 1 hr at 37°C as previously described (7) to reduce carry-over DNA. Cells were

exposed to the DNase-treated virus for 2h at 37°C, washed, cultured under standard conditions, and analyzed as described below.

The targets for HIV-1 infection were HTLV-I-carrying MT-2 cells (23), which are highly susceptible to infection with wild-type HIV-1 (13). It was found in preliminary experiments that MT-2 cells allow a reproducible distinction between *vif*$^+$ and *vif*-deficient infections, an important consideration given the variable requirement for *vif* during HIV-1 infection of different T cell lines (10). Table 1 shows the results of a representative experiment in which infection with *vif*$^+$ and *vif*-deficient viruses in MT-2 and CEM-SS cells were compared. Infection of MT2 cells by KS282 virus was slow, poorly productive, and non-cytopathic; in contrast, N1T-A replicated in these cells rapidly and caused massive cytolysis within five days of infection. The difference between *vif*$^+$ and *vif*-deficient infections at the same viral dose was much less pronounced in CEM-SS cells (Table 1), consistent with other studies which showed marked differences in the response of various T cell lines to *vif*-deficient infection (10). These results indicate that MT2 cells complement poorly, if at all, the *vif*-deficient phenotype of HIV-1, and thus are suitable for studies on the rate-limiting steps of *vif*-deficient virus infection.

MT-2 cells were infected with N1T-A or KS282 at different doses and examined for the kinetics and levels of HIV-1 DNA accumulation by quantitative polymerase chain reaction (PCR) (15) for the HIV-1 *gag* (Figure 2) and, in a separate experiment, *vif* regions (Figure 3). The *vif* primers were designed to flank the 35bp deletion in KS282 *vif* (30), allowing a clear distinction between the *vif*-defective (KS282) and wild-type (N1T-A) HIV-1 DNA (Figure 3). To allow quantitation of HIV-1 DNA present in cell

lysates, the amount of DNA present in lysates was first standardized using densitometric analysis of autoradiograms, by the β-globin DNA content to 10,000 cell-equivalents per assay system. Under our PCR conditions, this allowed reproducible detection of HIV-1 DNA within the range of 20-2000 *gag* DNA equivalents, using lysates of known numbers of ACH-2 cells (which carry 1-2 copies of HIV-1 DNA per cell, reference 6) as a reference. When the *vif*-defective and wild-type viruses were used at the same dose, an approximate MOI of 0.5 (0.4-0.5 pg p24 per cell), both *vif*-deficient and *vif*$^+$ infected cells showed detectable HIV-1 DNA signals within 2-5 hours after infection, and in both infections viral DNA increased to an intermediate plateau 10 hours after infection. However, the amount of viral DNA at this time as determined by densitometric analysis of autoradiograms, was about 5-7 fold lower in *vif*-deficient and *vif*$^+$ infection, and it remained stable at this level (about 150 *gag*-region DNA molecules per 10,000 cells) throughout the 10 day period of sampling. In contrast, the amount of viral *gag*-region DNA in MT-2 cells infected with the same dose of N1T-A virus increased rapidly, from about 1000 molecules per 10,000 cells at 10 hours to at least 2 x $10^4$ molecules per 10,000 cells on day 5 (Figure 2). Analysis of the *vif*-region DNA in the repeat experiment revealed a similar trend (Figure 3). Consistent with the observed patterns of viral DNA accumulation, and our previous studies (22, 30), N1T-A infection in MT-2 cells was rapid, and highly productive; KS282 replicated slowly (legend to Figure 2). The expression of complete KS282 DNA at 2-10 hours after infection confirms previous results which show that *vif*-deficient HIV-1 fuses with CD4$^+$ T cells (22) and indicates that some of the internalized viral nucleocapsids proceed through the step of reverse transcription.

It was important to verify that the autoradiography

results shown in Figures 2 and 3 represented newly synthesized HIV-1 DNA. This was demonstrated by including two negative controls to determine the baseline PCR signal, and by analyzing cells kinetically, starting at early times after infection, when minimal DNA synthesis is expected. When MT-2 cells were incubated with the DNase-treated virus at 0°C but not at 37°C, or infected with heat-inactivated virus at 37°C, PCR analysis revealed little or no HIV-1 DNA (Figures 2 and 3). This indicated that the DNase treatment of viral stocks in our experiments removed most of the carry-over viral DNA (7), resulting in low background PCR signal in the analyses shown. Analysis of viral DNA in MT-2 cells infected with N1T-A or KS282 viruses revealed very low levels of the PCR-detectable HIV-1 DNA at 2 or 5 hours after infection compared to later time points (Figures 2 and 3), further indicating that the HIV-1 DNA detected was synthesized *de novo*.

The results of infections with the same dose of N1T-A and KS282 (Figure 2 and Figure 3) indicated that the inefficient replication of KS282 in MT-2 cells (Table 1) correlates with lower levels (compared to N1T0A infection) of viral DNA at an early stage (0-10 hours) after infection, as well as with the slow accumulation of viral DNA during the infection expansion stage (10 hours to 3 days). Viral DNA detected during the first 12 hours of infection arises by reverse transcription from input viral RNA and it represents the first cycle of infection with a given dose of virus (17, 27, 28). The subsequent DNA accumulation is believed to be the product of repeated cycles of infection and of virus spread to uninfected cells (17, 27, 28). To determine whether the block to KS282 replication in MT-2 cells infected at an MOI of 0.5 is due to low levels of the early viral DNA, the amount of viral DNA present at 10 hours after infection was used to standardize input KS282 virus with

N1T-A of an MOI of 0.5. As shown in Figure 2, infection of MT-2 cells with KS282 at 2.24 pg per cell (MOI of about 2.5) yielded the same *gag* DNA levels at 10 hours post-infection as in cells infected with 0.39 pg/cell of N1T-A, that is, about 1000 gag-region DNA molecules per 10,000 cells. Thus, 5-fold more input KS282 than N1T-A allowed us to achieve an equal "intracellular titer" of the two viruses. In spite of this, KS282 infection proceeded slowly and the KS282 viral DNA levels remained stable throughout the 10-days of observation (Figure 2). A similar outcome of infection was obtained when the dose of N1T-A was reduced to 0.08 pg/cell, which brought the N1T-A DNA levels at 10 hours to less than 100 copies per 10,000 cells, below those found in KS282 infection at 0.45 pg/cell (Figure 2). It is noteworthy that in this case, in spite of the low dose of input N1T-A virus, N1T-A DNA accumulation and virus production accelerated rapidly between 3 and 5 days after infection, while KS282 DNA and virus production increased slowly regardless of the higher input viral dose and higher DNA levels at 10 hours (Figure 2). The results of these experiments suggest that the inefficient infection of MT-2 cells with a *vif*-defective virus cannot be attributed simply to a difference in input viral titers. It is also unlikely that the observed phenotype of *vif*-deficient infection is due to some unusual characteristics of MT-2 in terms of its susceptibility to HIV-1 binding, entry, or replication. To the contrary, MT-2 cells are permissive to highly productive infection with a *vif*-expressing HIV-1 (13), even when the virus inoculum is low (Figure 2).

Taken together, the data identify viral DNA synthesis as an early step of the HIV-1 life cycle which distinguishes *vif*-deficient from *vif*$^+$ infection in T cells. Two phases of restriction in the replication of *vif*-deficient virus have been revealed here, corresponding to two phases of HIV-1 infection. One is a deficiency in the first cycle

of infection, as visualized by the relatively inefficient synthesis of DNA from input viral RNA. That this deficiency occurs in spite of using virus produced in CEM-SS cells, in which *vif*-defective KS282 replicates relatively well (Table 1), indicates that phenotype complementation of *vif* mutant progeny virus found in some T cell lines (10) is not complete in CEM-SS cells. In addition, the *bona fide* MT-2-derived progeny virus has a major defect in the expansion phase of infection, as shown by the lack of significant increase in viral DNA copy number several days after initial exposure to KS282. Similar to the first cycle of infection, this defect is probably caused by inefficient synthesis of progeny KS282 viral DNA, impeding the multiple cycles of infection required for expansion of infection (17, 27, 28, 9, 22, 30). The defect in cell to cell spread of N1T-E-derived *vif* mutants (22) may further contribute to the observed poor infection and viral DNA synthesis in MT-2 cells.

The reasons for the apparently inefficient DNA synthesis in *vif*-deficient infection are presently unclear. Consistent with an early hypothesis (9, 34), *vif*-deficient virus preparations might contain a high proportion of defective viral particles which are incapable of either efficient internalization or reverse transcription after entry into cells in which the requirement for *vif*$^+$ particles is high. Since *vif*-defective HIV-1 fuses efficiently with target T cells (22), the purported defect at entry may involve post-membrane fusion events such as viral uncoating, transit of viral nucleocapsids through the cytoskeleton, or disassembly of viral core leading to reverse transcription.

vif$^-$ HIV does not infect human peripheral blood lymphocytes, but recent experiments have shown that vif$^-$ HIV does successfully infect human peripheral blood

macrophages, and *vif*⁻ HIV-infected primary macrophages produce *vif*⁻ HIV (data not shown). Therefore, *vif*⁻ HIV would be expected to infect a subject but would not be expected to infect and replicate in peripheral blood lymphocytes.

Table 1

Biological activity of *vif*-defective and *vif*-expressing HIV-1 in MT-2 and CEM cells.

| | HIV-1 antigen expression | | | | | |
|---|---|---|---|---|---|---|
| Supernatant Virus | CPE | | IF (% positive cells) | | p24 core antigen (ng/ml) | |
| | MT-2 | CEM | MT-2 | CEM | MT-2 | CEM |
| NIT-A | 3+ | 3+ | 96* | 73 | 350 | 310 |
| KS282 | ± | 2+ | 2.5 | 35 | 2 | 500 |
| None | - | - | <0.01 | <0.01 | <2 | <2 |

Cells were infected with virus at 1pg p24 per cell (MOI of 1) and evaluated 9 days after infection (6 days in the system marked with*) as described in the text and elsewhere (37). CPE: cytopathic effects (cell fusion and

lysis); 3+ represents the maximum CPE observed (more than 5 giants cells of ≥ 5 fused cells in a field of approximately 100 cells by day 2 and more than 80% of cells lysed by day 9). IF: indirect immunofluorescence assay for HIV-1 antigens, includes living and dead cells. Supernatant HIV-1 p24 core antigen levels were determined using Coulter HIV Ag assay (Coulter Immunochemicals, Hialeah, FL.) according to the manufacturer's instructions.

Replication of *vif*-negative human immunodeficiency virus type 1 (HIV-1) is attenuated in certain cell lines and highly impaired in peripheral blood lymphocytes *in vitro*. To determine whether intact *vif* is positively selected during natural HIV-1 infection and to determine *vif* sequence variability, we employed polymerase chain reaction amplification, cloning, and sequencing to investigate the *vif* region of replicating virus in short term passage HIV-1 primary isolates from five asymptomatic individuals and from five persons with AIDS. A total of 46 *vif* clones were obtained and analyzed. Recombinant proviruses were constructed using selected *vif* clones and were found to be fully infectious. We found that 38 of the 46 clones sequenced carried open *vif* reading frames and that there was a low degree of heterogeneity of *vif* genes within individual isolates or among isolates from different donors. The cysteines previously found to be essential for *vif* protein function were conserved in all clones. There was no correlation between disease status and presence of intact *vif*. A phylogenetic tree constructed of all available *vif* nucleotide sequences resulted in a virus grouping similar to those of *gag* or *env*. The conservation of the *vif* open reading frame and its limited variability are consistent with a role for *vif* in natural HIV-1 infection (data not shown).

*vif* is required for productive, cytopathic HIV-1 infection of peripheral blood mononuclear cells (PBMC). Single-cycle infection of PBMC with a *vif* deletion mutant was achieved using phenotypically complemented virus. Progeny virus was produced and was sedimentable but was noninfectious in PBMC. PCR amplification of PBMC exposed to PBMC-derived *vif* mutant revealed that viral DNA synthesis was initiated with about a 10-fold reduction compared to wild type virus, but that completion of DNA synthesis was profoundly impaired; fewer than five copies of full length viral DNA were found per 150,000 cells. Protein analysis of progeny virions by radioimmunoprecipitation with AIDS patient serum or with a monoclonal antibody reactive with HIV-1 core proteins revealed high levels of a 55 kilodalton protein, in addition to p24, in *vif*-negative virions produced by PBMC but not by SUpT1 cells, and not in wild-type viral particles from either source (data not shown). We suggest that the presence of putative unprocessed $Pr55^{gag}$ in PBMC-derived *vif*-negative virions contributes to the greatly diminished infectivity of this virus in PBMC by reducing the ability of viral nucleocapsids to efficiently complete reverse transcription.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

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Potash, Mary Jane

(ii) TITLE OF INVENTION: HIV vif-RELATED COMPOSITIONS, AND PROPHYLACTIC AND THERAPEUTIC USES THEREOF

(iii) NUMBER OF SEQUENCES: 2

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 542 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATGGAAAACA GATGGCAGGT GATGATTGTG TGGCAAGTAG ACAGGATGAG GATTAGAACA 60

TGGAAAAGTT TAGTAAAACA CCATATGTAT GTTTCAGGGA AAGCTAGGGG ATGGAATAAG 120

TTCAGAAGTA CACATCCCAC TAGGGGATGC TAGATTGGTA ATAACAACAT ATTGGGGTCT 180

GCATACAGGA GAAAGAGACT GGCATTTGGG TCAGGGAGTC TCCATAGAAT GGAAGAAAAA 240

GAGATATAGC ACACAAGTAG ACCCTGAACT AGCAGACCAA CTAATTCATC TGTATTACTT 300

TGACTGTTTT TCAGACTCTG CTATAAGAAA GGCCTTATTA GGACACATAG TTAGCCCTAG 360

GTGTGAATAT CAAGCAGGAC ATAACAAGGT AGGATCTCTA CAATACTTGG CACTAGCAGC 420

ATTAATAACA CCAAAAAGGA TAACGCCACC TTTGCCTAGT GTTACAAAAC TGACAGAGGA 480

TAGATGGAAC AAGCCCCAGA AGACCAAGGG CCACAGAGGG AGCCACACAA TGAATAGACA 540

CT 542

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 577 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATGGAAAACA GATGGCAGGT GATGATTGTG TGGCAAGTAG ACAGGATGAG GATTAGAACA 60

TGGAAAAGTT TAGTAAAACA CCATATGTAT GTTTCAGGGA AAGCTAGGGG ATGGTTTTAT 120

AGACATCACT ATGAAAGCCC TCATCCAAGA ATAAGTTCAG AAGTACACAT CCCACTAGGG 180

GATGCTAGAT TGGTAATAAC AACATATTGG GGTCTGCATA CAGGAGAAAG AGACTGGCAT 240

TTGGGTCAGG GAGTCTCCAT AAAATGGAGG AAAAAGAGAT ATAGCACACA AGTAGACCCT 300

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AGAAAGGCCT TATTAGGACA CATAGTTAGC CCTAGGTGTG AATATCAAGC AGGACATAAC 420

AAGGTAGGAT CTCTACAATA CTTGGCACTA GCAGCATTAA TAACACCAAA AAAGATAAAG 480

CCACCTTTGC CTAGTGTTAC GAAACTGACA GAGGACAGAT GGAACAAGCC CCAGAAGACC 540

AAGGGCCACA GAAGGAGCCA CACAATGAAT GGACACT 577

What is claimed is:

1. A vaccine which comprises an effective immunizing amount of *vif*⁻ HIV and a pharmaceutically acceptable carrier.

2. A method of reducing the likelihood of a non-HIV-infected subject's becoming infected with HIV which comprises immunizing the non-HIV-infected subject with the vaccine of claim 1 so as to thereby reduce the likelihood of the subject's becoming infected with HIV.

3. A composition which comprises an effective amount of a nucleic acid molecule encoding *vif*⁻ HIV capable of being expressed in a suitable host cell, and a pharmaceutically acceptable carrier.

4. A method of reducing the likelihood of a non-HIV-infected subject's becoming infected with HIV which comprises administering to the non-HIV-infected subject an amount of the composition of claim 3 effective to reduce the likelihood of the subject's becoming infected with HIV.

5. A composition which comprises an effective amount of a recombinant non-HIV virus capable of infecting a suitable host cell, said recombinant virus comprising a nucleic acid molecule encoding *vif*⁻ HIV and capable of being expressed in the suitable host cell, and a pharmaceutically acceptable carrier.

6. The composition of claim 5, wherein the recombinant non-HIV virus is a retrovirus and the nucleic acid molecule is an RNA molecule.

7. A method of reducing the likelihood of a non-HIV-infected subject's becoming infected with HIV which comprises administering to the non-HIV-infected subject an amount of the composition of claim 5 effective to reduce the likelihood of the subject's becoming infected with HIV.

8. A composition which comprises an effective amount of a recombinant non-HIV virus capable of infecting a suitable host cell, said recombinant virus comprising a nucleic acid molecule (a) encoding an anti-sense oligonucleotide molecule capable of specifically binding to an mRNA molecule encoding HIV *vif* protein, at the portion thereof encoding the HIV *vif* protein, so as to prevent translation of the mRNA molecule, and (b) capable of being expressed in the suitable host cell, and a pharmaceutically acceptable carrier.

9. The composition of claim 8, wherein the virus is a retrovirus.

10. A method of reducing the likelihood of a non-HIV-infected subject's becoming infected with HIV which comprises administering to the non-HIV-infected subject an amount of the composition of claim 8 effective to reduce the likelihood of the subject's becoming infected with HIV.

11. A method of treating an HIV-infected subject which comprises administering to the HIV-infected subject an amount of the composition of claim 8 effective to treat the subject.

12. A method of treating an HIV-infected subject which comprises:

(a) obtaining a sample of hematopoietic stem cells from the HIV-infected subject;

(b) culturing the sample in vitro, thereby producing cultured hematopoietic stem cells;

(c) introducing into the cultured hematopoietic stem cells so produced a nucleic acid molecule (a) encoding an anti-sense oligonucleotide molecule capable of specifically binding to an mRNA molecule encoding HIV *vif* protein, at the portion thereof encoding the HIV *vif* protein, so as to prevent translation of the mRNA molecule, and (b) capable of being expressed in the cultured hematopoietic stem cells; and

(d) introducing the resulting cultured hematopoietic stem cells into the HIV-infected subject under conditions permitting the resulting cultured hematopoietic stem cells to reconstitute the HIV-infected subject's hematopoietic system, so as to thereby treat the HIV-infected subject.

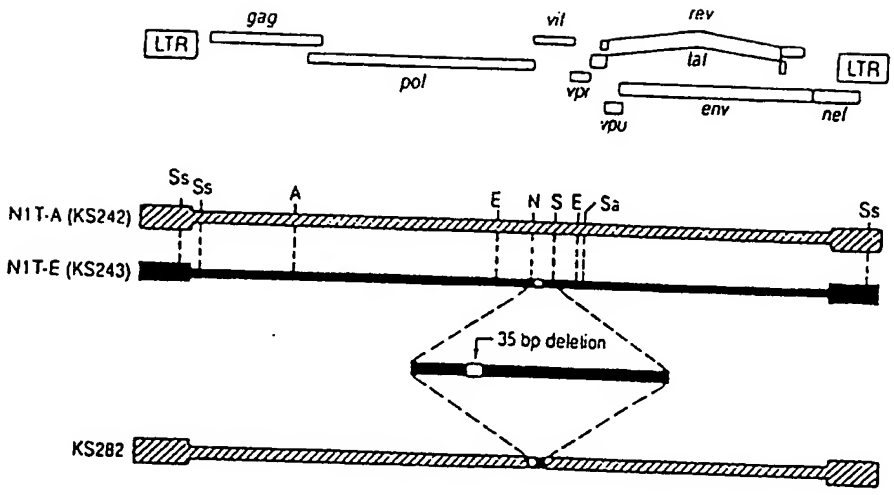
13. A composition which comprises an effective amount of a recombinant non-HIV virus capable of infecting an HIV-infected cell, said recombinant virus comprising a nucleic acid molecule (a) encoding a non-functional mutant HIV *vif* protein capable of competitively inhibiting the function of HIV *vif* protein in the HIV-infected cell, and (b) capable of being expressed in the HIV-

infected cell, and a pharmaceutically acceptable carrier.

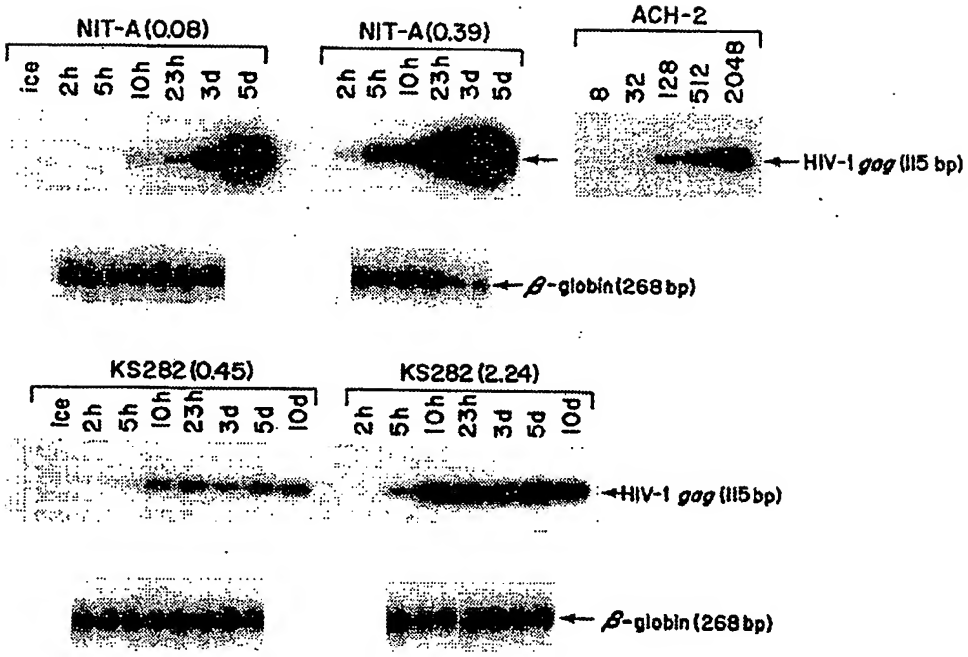
14. The composition of claim 13, wherein the recombinant non-HIV virus is a retrovirus.

15. A method of treating an HIV-infected subject which comprises administering to the HIV-infected subject an amount of the composition of claim 13 effective to treat the HIV-infected subject.

# FIGURE 1


LTR
gag
pol
vif
vpr
vpu
rev
tat
env
nef
LTR
Ss
Ss
A
E
N
S
E
Sa
Ss
N1T-A (KS242)
N1T-E (KS243)
35 bp deletion
KS282

# FIGURE 2


NIT-A(0.08)
ice
2h
5h
10h
23h
3d
5d
NIT-A(0.39)
2h
5h
10h
23h
3d
5d
ACH-2
8
32
128
512
2048
HIV-1 gag (115 bp)
β-globin (268 bp)
KS282(0.45)
ice
2h
5h
10h
23h
3d
5d
10d
KS282(2.24)
2h
5h
10h
23h
3d
5d
10d
HIV-1 gag (115 bp)
β-globin (268 bp)

# FIGURE 3

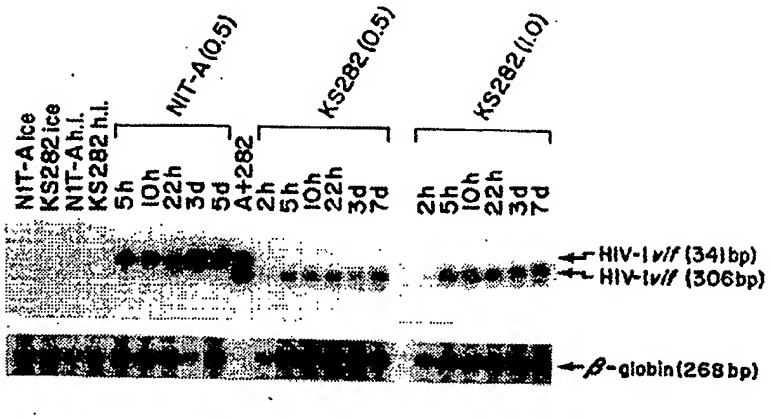
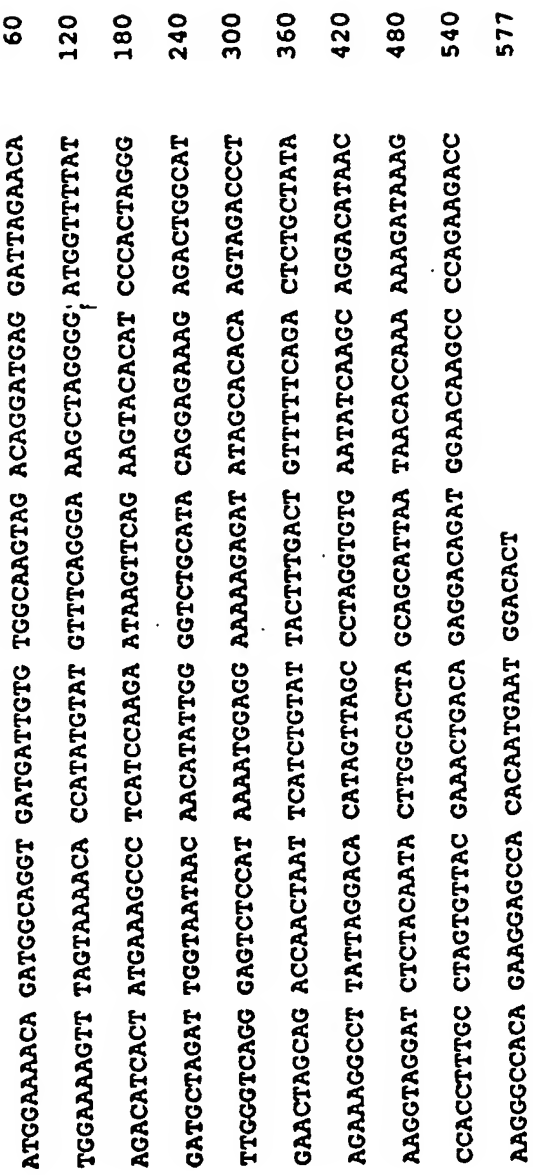

NIT-A ice
KS282 ice
NIT-A h.i.
KS282 h.i.
NIT-A (0.5)
KS282 (0.5)
KS282 (1.0)
5h
10h
22h
3d
5d
A+282
2h
5h
10h
22h
3d
7d
2h
5h
10h
22h
3d
7d
HIV-1 vif (341bp)
HIV-1 vif (306bp)
β-globin (268 bp)

FIGURE 4


ATGGAAAACA GATGGCAGGT GATGATTGTG TGGCAAGTAG ACAGGATGAG GATTAGAACA 60
TGGAAAAGTT TAGTAAAACA CCATATGTAT GTTTCAGGGA AAGCTAGGGG ATGGTTTTAT 120
AGACATCACT ATGAAAGCCC TCATCCAAGA ATAAGTTCAG AAGTACACAT CCCACTAGGG 180
GATGCTAGAT TGGTAATAAC AACATATTGG GGTCTGCATA CAGGAGAAAG AGACTGGCAT 240
TTGGGTCAGG GAGTCTCCAT AAAATGGAGG AAAAGAGAT ATAGCACACA AGTAGACCCT 300
GAACTAGCAG ACCAACTAAT TCATCTGTAT TACTTTGACT GTTTTTCAGA CTCTGCTATA 360
AGAAAGGCCT TATTAGGACA CATAGTTAGC CCTAGGTGTG AATATCAAGC AGGACATAAC 420
AAGGTAGGAT CTCTACAATA CTTGGCACTA GCAGCATTAA TAACACCAAA AAAGATAAAG 480
CCACCTTTGC CTAGTGTTAC GAAACTGACA GAGGACAGAT GGAACAAGCC CCAGAAGACC 540
AAGGGCCACA GAAGGAGCCA CACAATGAAT GGACACT 577

# FIGURE 5

```
TTCAGAAGTA CACATCCCAC TAGGGGATGC TAGATTGGTA ATAACAACAT ATTGGGGTCT  180
GCATACAGGA GAAAGAGACT GGCATTTGGG TCAGGGAGTC TCCATAGAAT GGAAGAAAAA  240
GAGATATAGC ACACAAGTAG ACCCTGAACT AGCAGACCAA CTAATTCATC TGTATTACTT  300
TGACTGTTTT TCAGACTCTG CTATAAGAAA GGCCTTATTA GGACACATAG TTAGCCCTAG  360
GTGTGAATAT CAAGCAGGAC ATAACAAGGT AGGATCTCTA CAATACTTGG CACTAGCAGC  420
ATTAATAACA CCAAAAAGGA TAACGCCACC TTTGCCTAGT GTTACAAAAC TGACAGAGGA  480
TAGATGGAAC AAGCCCCAGA AGACCAAGGG CCACAGAGGG AGCCACACAA TGAATAGACA  540
CT                                                                 542
```

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/09313

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :A61K 39/12, 39/21; C12N 7/04, 15/49
US CL :424/187.1, 208.1; 435/69; 514/44; 536/23.72, 24.5
According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/187.1, 208.1; 435/69; 514/44; 536/23.72, 24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Medline, Biosis, Embase, Derwent
search terms: HIV, vif, antisense, transdominant, vaccine

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | AIDS Research and Human Retroviruses, Volume 8, Number 3, issued March 1992, R.C. Desrosiers, "HIV with Multiple Gene Deletions as a Live Attenuated Vaccine for AIDS", pages 411-421, see entire document, especially Tables 6 and 12. | 1-4 |
| X | WO, A, 91/05864 (HAYNES ET AL.) 02 May 1991, see entire document, especially page 16. | 5-7 |
| Y | EP, A, 0,403,333 (MONCANY ET AL.) 19 December 1990, see entire document. | 8-12 |
| Y | EP, A, 2,647,809 (MONCANY ET AL.) 02 June 1989, see entire document. | 8-12 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 17 NOVEMBER 1994 | 09 DEC 1994 |
| Name and mailing address of the ISA/US<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231<br>Facsimile No. (703) 305-3230 | Authorized officer<br>Micahel S. Tuscan, Ph.D.<br>Telephone No. (703) 308-0196 |

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/09313

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | Proceedings of the National Academy of Sciences USA, Volume 90, issued February 1993, P.F. Torrence et al., "Targeting RNA for Degradation with a (2'-5')Oligoadenylate-Antisense Chimera", pages 1300-1304, see entire document. | 8-12 |
| Y | Nucleic Acids Research, Volume 19, Number 7, issued 1991, K. Rittner et al., "Identification and Analysis of Antisense RNA Target Regions of the Human Immunodeficiency Virus Type I", pages 1421-1426, see entire document. | 8-12 |
| Y | WO, A, 90/07936 (GOLDSMITH ET AL.) 26 July 1990, see entire document. | 8-12 |